From DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

MYELOID CELLS IN EXPERIMENTAL NEUROPATHOLOGIES

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Stockholm 2019

Published by Karolinska Institutet.
Printed by Eprint AB 2019

ISBN 978-91-7831-408-9

Myeloid Cells in Experimental Neuropathologies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The first innate immune cells, most importantly for this thesis macrophages, microglia and eosinophils, were described more than 100 years ago and only now do we begin to understand more about their development, functions and properties.
So while elimination of pathogens through phagocytosis (macrophages/microglia) or degranulation (eosinophils) were long considered as their main functions, research during the last decades has made it clear that innate immune cells do much more than maintain homeostasis in an organism.

Monocytes were considered the progenitors of all macrophages, migrating from the bloodstream into tissues in order to colonize them. A groundbreaking discovery changed our view of tissue macrophage development, proving that they are established during embryonic development and that they maintain themselves during homeostasis in tissues without input from circulating monocytes. Conversely, monocyte-derived macrophages primarily respond to pathogens or injury. Eosinophils were for a long time disregarded to have major functions in diseases other than allergies or parasitic infections. However, we now know that eosinophils not only play important roles in many diseases, including cancer, but also during homeostasis.
In this thesis I describe how, in the event of significant loss of tissue macrophages, a tissue can be repopulated by rapid proliferation of resident cells in addition to infiltration from monocytes from the periphery. I have thus primarily studied microglia in the CNS niche under both homeostatic conditions and during disease states.

Our work identified TGF-β as a major signaling pathway involved in niche homeostasis and macrophage functional integration into the CNS. Loss of TGF-β signaling or an inability to respond to it led to deregulation of microglial function and infiltrating macrophage function, causing spontaneous initiation of a fatal motor disease characterized by demyelination and damage to neurons.

We further established a role for eosinophils in a mouse model of Glioblastoma multiforme brain tumors, in which depletion of eosinophils led to significantly longer survival of tumor-bearing mice, which is in contrast to the current belief that macrophages are the most important and major infiltrating immune cell in most solid tumors. As a last approach, we used siRNA-loaded nanoparticles to reduce target gene expression of importance in tumor development in a mouse model of melanoma. Targeted delivery of therapeutic agents is one of the main obstacles in the treatment of cancer. Nanoparticles can be engineered to specifically target tumor cells or cells in the tumor vicinity and can be loaded with virtually any siRNA, without majorly affecting healthy cells and tissue. Using two delivery systems we demonstrated feasibility and efficacy of this approach.

In summary, in this thesis I highlight the importance of myeloid immune cells in health and disease and how targeting them could be of potential therapeutic interest in the future.

LIST OF SCIENTIFIC PAPERS

I. Harald Lund, **Melanie Pieber**, Roham Parsa, Jinming Han, David Grommisch, Ewoud Ewing, Lara Kular, Maria Needhamsen, Alexander Espinosa, Emma Nilsson, Anna K. Överby, Oleg Butovsky, Maja Jagodic, Xing-Mei Zhang, Robert A. Harris. *Competitive repopulation of an empty microglial niche yields functionally distinct subsets of microglia-like cells.*
Nature Communications. 19;9(1):4845 **(November 2018)**

II. Harald Lund, **Melanie Pieber**[*], Roham Parsa[*], David Grommisch, Ewoud Ewing Lara Kular, Maria Needhamsen, Sabrina Ruhrmann, André Ortlieb Guerreiro Cacais, Oleg Butovsky, Maja Jagodic, Xing-Mei Zhang, Robert A. Harris. *Fatal demyelinating disease is induced by monocyte-derived macrophages in the absence of TGF-β signaling.*
Nature Immunology. 19(5), 1-7 **(May 2018)**

III. **Melanie Pieber**, Roham Parsa[*], Harald Lund[*], Elizabeth A. Jacobsen, Robert A. Harris, Xing-Mei Zhang. *Eosinophil depletion as a novel treatment in a mouse model of Glioblastoma multiforme.*
Manuscript

IV. **Melanie Pieber**, Keying Zhu, Vigneshkumar Rangasami, Oommen Podiyan Oommen, Myriam Aouadi, Robert A. Harris. *Assessment of nanoparticle siRNA delivery in vitro and in vivo for the treatment of tumors in mouse models of melanoma and Glioblastoma multiforme.*
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

MPS	Mononuclear phagocyte system
EMP	Erythro-myeloid precursor
CSF/CSFR	Colony stimulating factor/Colony stimulating factor receptor
HSC	Hematopoietic stem cell
RPM	Red pulp macrophage
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
GMP	Granulocyte-macrophage progenitor
cMoP	Common monocyte progenitor
CDP	Common DC progenitor
DC	Dendritic cell
iNOS	Induced nitric oxide synthase
CNS	Central nervous system
PRR	Pattern recognition receptor
PAMP	Pathogen associated molecular pattern
DAMP	Danger associated molecular pattern
SVZ	Subventricular zone
SGZ	Subgranular zone
DT/DTR	Diphteria toxin/Diphteria toxin receptor
i.c.v	Intracerebroventricular
EoMP	Eosinophil/Mast cell progenitor
TATE	Tumor associated tissue eosinophilia
FDA	Food and drug administration
WHO	World health organization
GBM	Glioblastoma multiforme
BBB	Blood brain barrier
TAM	Tumor associated macrophage
RNAi/siRNA	RNA interference/small interfering RNA
TGF-β	Transforming growth factor beta
CQ-siRNA-HA	Chloroquine-siRNA-Hyaloronic acid

1 INTRODUCTION

1.1 INNATE IMMUNE SYSTEM

Innate immunity was first introduced in 1989 by Charles Janeway Jr., who suggested a general theory of innate immune recognition by mechanisms of pattern recognition, and proposed that the innate immune system controls the adaptive immune system.
In defense against pathogens the innate immune system is the first line of defense and it is a semi-specific system to keep an organism in homeostasis[1]. The major constituents include surface barriers (most importantly the skin), humoral defense (macromolecules found in extracellular fluids) and cellular defense (innate leukocytes)[2].

External structures present the first barrier for an invading pathogen, isolating the internal environment from external influences and preventing the spread of microorganisms and infection. Epithelial cells connected by tight junctions form complex structures throughout the organism to prevent pathogens from entering. Is this structure damaged, for example by mechanical forces or insect bites, pathogens are able to enter the organism and the second and third barriers will come into play.
Humoral immunity is commonly referred to as the second barrier of innate immunity and comprises of macromolecules found in extracellular fluids. Many different substances have been discovered over the years, with the complement proteins first being discovered by Hans Buchner (1890) and Paul Ehrlich (1892). These are substances in the serum capable of killing pathogens through opsonization, formation of pores in the membrane (membrane attack complex) and induction of an inflammatory response, leading to lysis of the pathogen. Other humoral components are for example cytokines (which mediate communication between immune cells), opsonins (that coat outer membranes of foreign substances and enhance phagocytosis), anti-toxins (neutralize toxins produced by invading bacteria) and antibodies (part of the adaptive immune response).
Cellular innate immunity comprises of various different cell types and is considered the third line of defense of the innate immune system. Innate leukocytes include γδ T-cells, natural killer cells, mast cells, basophils and eosinophils, as well as phagocytic cells including neutrophils, dendritic cells and monocytes/macrophages. All these different cell types function within the innate immune defense by identifying and eventually eliminating pathogens that can cause infections. Innate leukocytes can move freely, as they are commonly not associated with a specific tissue or organ, and can therefore easily detect, interact and clear cellular debris, pathogens or other foreign particles[3,4].

In this thesis I focus on two innate leukocytes, macrophages and eosinophils, and describe their development and their role in health and disease.

1.2 TISSUE RESIDENT MACROPHAGES

Ilya Metchnikoff first discovered macrophages in the late 19th century. Metchnikoff, who is now considered to be the father of modern immunology, discovered phagocytic cells in his studies of frogs and starfish.

"*I rather believe that the essence of an inflammation lies in the phagocyte attack of solid pathogenic substances, be it a weakened or dead cell, a bacterium or any other foreign body*." Ilya Metchnikoff, 1883

This sentence from his 1883 publication distinctly places phagocytes as the major players in defense against pathogens in a multicellular organism. Phagocytic cells are conserved among a huge number of species, highlighting their importance. The discovery of phagocytosis by Ilya Metchnikoff was awarded with the Nobel Prize in Physiology and Medicine in 1908[5,6].

A lot of research has been conducted during the last decades on the origin of macrophages. One of the major influencers in the field of macrophage biology was Ralph van Furth. He proposed in the 1960s that all tissue macrophages originate and are replenished from circulating monocytes in the blood, coining the term 'mononuclear phagocyte system (MPS)'[7]. However, with advancement in genetic techniques, studies performed in recent years came to a different conclusion and challenged the traditional MPS that van Furth characterized. It is now known that tissue macrophages are actually established during embryogenesis and persist in adulthood, without (or with limited) input from circulating monocytes[8,9,10,11,12] and that circulating monocytes can, in addition, differentiate into macrophages upon inflammatory stimulation[39].

1.2.1 Development of Tissue Resident Macrophages

Tissue resident macrophages have crucial functions during development, tissue homeostasis and resolution of inflammation and already develop during the early stages of hematopoiesis in the embryo. The first stage, also called primitive hematopoiesis, develops from the posterior plate mesoderm in the blood islands of the extra-embryonic yolk sac at around E7.0, giving rise to primitive erythroblasts, megakaryocytes and macrophages[13]. These early macrophages are independent of the transcription factor MYB, but rely on PU.1, which is in contrast to later macrophages that develop during definitive hematopoiesis. The pattern of macrophage differentiation is distinctive in that they do not go through a monocyte intermediate stage, which is characteristic for adult bone marrow-derived macrophages[14]. The second stage, the 'transient definitive stage' of hematopoiesis, leads to the formation of hematopoietic progenitors called erythro-myeloid precursors (EMPs), which arise from the yolk sac hemogenic endothelium between E8.0 and E8.5. These EMPs can develop into erythroid and myeloid cells, but do not have the potential to develop into lymphoid cells[15]. EMPs migrate to the fetal liver where they expand in numbers and differentiate into different cell types, including monocytes.

After a full blood circulation is developed in the embryo the third stage of hematopoiesis, the definitive stage, takes place (E10.5). A new wave of hematopoietic progenitors arises from the intraembryonic hemogenic endothelium in the aorta-, gonads-, mesonephros-region[16,17]. These precursors then colonize the fetal liver and the fetal bone marrow, eventually giving rise to adult bone marrow hematopoietic stem cells. In the embryo the fetal liver will become the major hematopoietic organ, containing progenitors of different origins and different potentials for differentiation. Together, these then will give rise to a functioning immune system.

Our understanding of macrophage development has drastically changed since van Furth proposed the MPS in the 1960s. It is now known that monocytes have no major contribution to most tissue macrophage pools (neither during homeostasis nor during inflammation), that adult tissue macrophages are derived from embryonic precursors that already seed in their specific tissues before birth, and that tissue macrophages maintain themselves in adulthood by self-renewal[14].

Embryonically-derived tissue macrophages require a mechanism that allows them to replenish old or apoptotic macrophages during adult life. On the one hand this could occur through tissue resident stem cell-like populations with a potential for asymmetric division or, conversely, through differentiated macrophages that have self-renewal potential. Evidence for the first scenario is currently lacking, but different fate mapping studies suggest that tissue macrophages can switch between terminal differentiation and proliferation, retaining the macrophage population in homeostasis. This self-renewal of tissue macrophages significantly differs from stem cell self-renewal since it results in the formation of two daughter cells that are identical, instead of asymmetrical division that takes place in stem cells. Self-renewal of tissue macrophages is dependent on CSF1 and CSF2 and is driven by the transcription factors MafB and cMaf[18,19,20].

Evidence for the ‘revised’ mononuclear phagocyte system came from various different fate mapping studies and simple observations. For example, monocytopenic animals (which have a reduced number of circulating monocytes in their blood) do not exhibit any differences in their tissue macrophage compositions, suggesting that monocyte input is not necessary for tissue macrophage renewal[20]. In addition, it was observed that in the absence of hematopoietic stem cells (HSC), yolk sac progenitors are still capable of giving rise to the main tissue resident macrophage populations in the spleen, the pancreas, the liver, the brain, the skin and the lung. Some tissue macrophage populations (lung, kidney) are of ‘chimeric’ origin, meaning they arise from HSC and yolk-sac progenitors[9].

Fate mapping studies make use of the fact that during embryonic development the expression of transcription factors differs between cells, which makes it possible to genetically label cells and all their progeny, either constantly (Cre) or with inducible (CreER) systems. For example, yolk-sac-derived macrophages develop independently of the transcription factor MYB, whereas definitive HSC-derived macrophages are dependent on this factor for their development and differentiation[6,9].

This knowledge can be used to track cells that are MYB^+ or MYB^-, respectively, at certain time points during development and into adulthood, making it possible to determine the origin of various adult cell populations.

1.2.2 Generic and Tissue Specific Functions of Macrophages

Macrophages are an essential part of the innate immune system and act as guards in tissues and the whole organism. They are specialized phagocytes and antigen presenting cells, neutralizing pathogens and communicating with other parts of the innate as well as adaptive immune systems to mount powerful responses against invaders. Tissue macrophages are mostly stationary cells that monitor their immediate environments using different sensory molecules such as scavenger receptors, pattern recognition receptors (for example Toll-like receptors (TLRs), RIG-I-like receptors, C-type lectin receptors and Nod-like receptors), nuclear hormone and cytokine receptors as well as adhesion molecules.

The 'guard' function of tissue macrophages is a universal feature of the macrophage cell, with only minor adjustments needed depending on the particular tissue in question. Even though macrophages look morphologically similar in all tissues they display highly distinct and characteristic gene expression signatures and epigenetic signatures. This means that, beyond their unifying functions, tissue macrophages have specific tasks associated with the homeostasis of the particular tissue of residence[21,22].

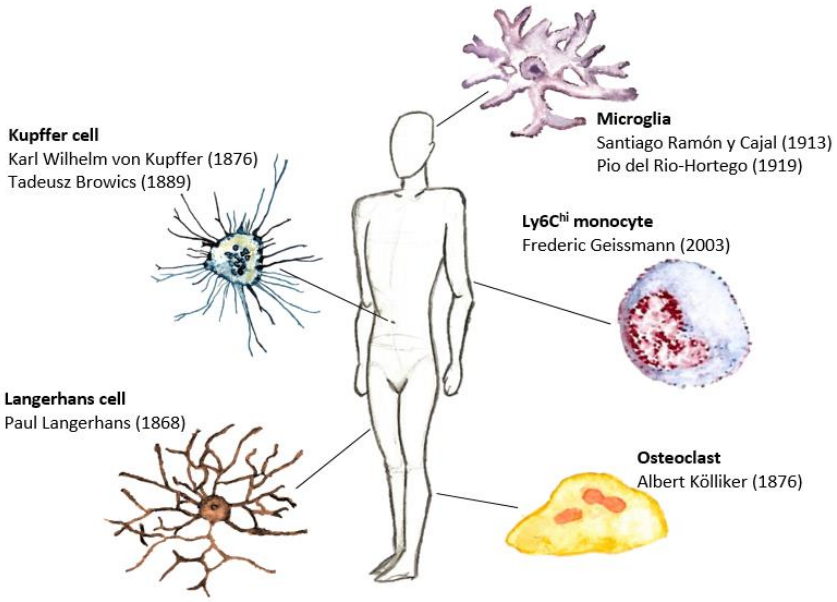

Figure 1: Examples of tissue macrophages and their discovery. Kupffer cell *(blue)*, Microglia *(pink)*, Monocyte *(blue-red)*, Osteoclast *(yellow)*, Langerhans cell *(brown)*.

In the lung, for example, alveolar macrophages are continuously exposed to microorganisms, pollutants and dust from the air we breathe. A major tissue-specific function of alveolar macrophages is to tolerate inhaled harmless stimuli, while preserving the ability of mounting an immune response against opportunistic pathogens. Alveolar macrophages are involved in acute immune responses and tolerance induction and clear excessive surfactant from the lungs by phagocytosis[23,24].
Macrophages in the liver, also called Kupffer cells, are specialized in detoxification and iron and cholesterol recycling. They do so by filtering the blood and therefore need to be highly phagocytic and have a high lysosomal activity. Kupffer cells form a barrier for pathogens to enter the systemic circulation and are excellent capturers of antigens and presenters to the environment[25].
Splenic macrophages reside in two different sites of the spleen. Red pulp macrophages (RPMs) that reside in the red pulp are specialized in uptake of hemoglobin and scavenging of senescent or damaged erythrocytes and recovering iron from these cells. Marginal zone macrophages are located in the white pulp of the spleen together with marginal metallophilic macrophages and clear pathogens from the systemic circulation as well as dead cells and debris[26,27].

1.3 MONOCYTE DERIVED MACROPHAGES

Monocytes are key players during inflammation and pathogen challenge. Monocytes are a highly plastic population of cells that can complement the classical tissue resident macrophage upon demand (during inflammation or infection) and exist as two major populations in humans and mice.

'Classical' monocytes have a half-life of about 20 hours in the blood, and 'non-classical' monocytes are longer lived and can exist over several days. Both populations are primarily present in the blood circulation, the spleen, the lung and the bone marrow[28,29].

1.3.1 Development of Monocytes

Monocytes are blood mononuclear cells with bean-shaped nuclei that are constantly produced in adults and arise from definitive HSCs. They are a conserved population of leukocytes in both mice and humans.
HSCs first develop into proliferative precursor cells, previously called 'monoblasts' and 'pro-monocytes'[30,31] and nowadays referred to as 'multipotent progenitors'. Multipotent progenitors (MPP) develop further into either 'common lymphocyte progenitors' (CLP) (which will give rise to T- and B-cells) or 'common myeloid progenitors' (CMP). The CMP lineage subdivides further into MEP ('megakaryocyte-erythrocyte progenitor'), which give rise to platelets and erythrocytes, and a GMP ('granulocyte-macrophage progenitor') branch[32,33].

GMP can either directly differentiate into basophils, eosinophils or neutrophils or differentiate into the ‘monocyte-macrophage DC progenitors’ (MDP), which have the potential to give rise to a ‘common DC progenitor’ (CDP) as well as ‘common monocyte progenitors’ (cMoP).

CDPs can only commit to become a dendritic cell and do not develop into monocytes or macrophages[34,35]. Monocytes solely derive from cMoP, which was identified in 2013 by Hettinger *et al.*[36]. The processes and transcription factors determining whether MDP decide to become a CDP and eventually give rise to dendritic cells or become a cMoP and give rise to monocytes are as yet not well understood, but it is clear that monocytes, macrophages and DCs are developmentally closely related and share similar functions in the body.

The development and survival of monocytes are entirely dependent on colony-stimulating factor 1 (CSF1 or MCSF), and mice that lack either CSF1 or its receptor CSF1R (CD115) are severely monocytopenic[37,38].

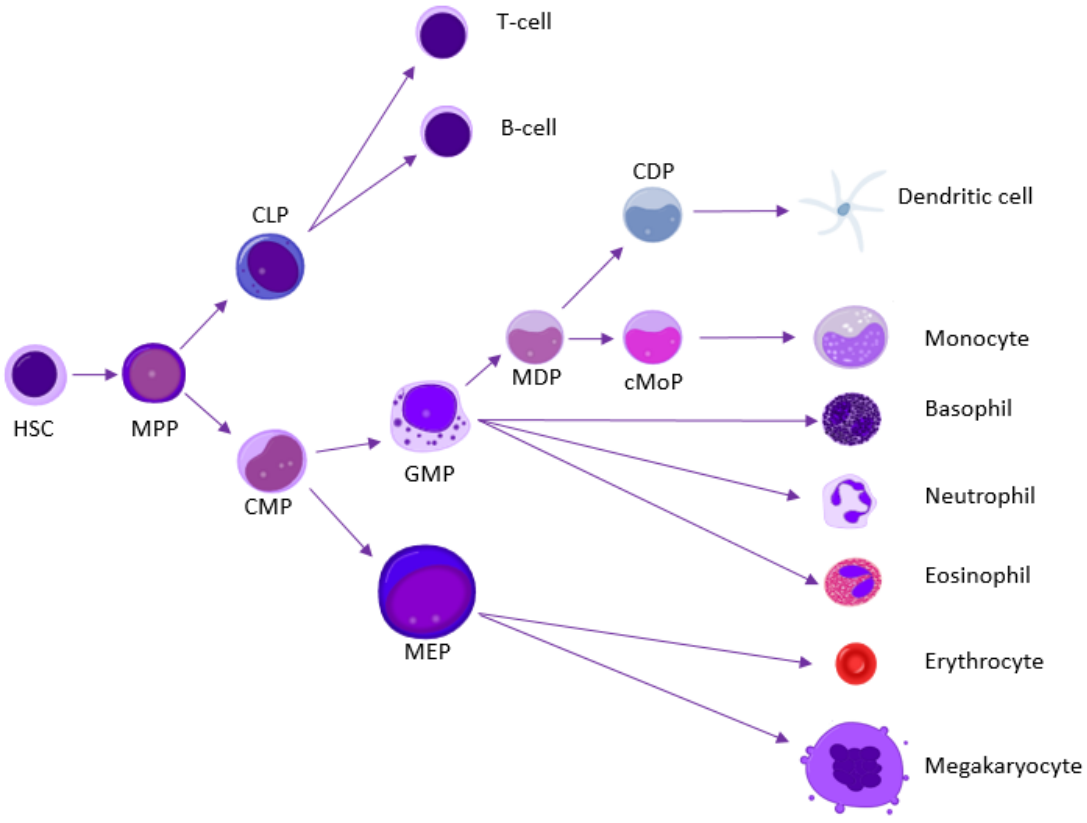

Figure 2: Classic model of hematopoiesis. HSC-Hematopoietic stem cell, MPP-multipotent progenitor, CLP-common lymphoid progenitor, CMP-common myeloid progenitor, MEP-megacaryocyte-erythrocyte progenitor, GMP-granulocyte-macrophage progenitor, MDP-monocyte-macrophage DC progenitor, cMoP-common monocyte progenitor, CDP-common DC progenitor.

Monocytes are described by their morphology, characteristic gene signatures and miRNA signatures and are currently classified into two main populations: 'classical' or 'inflammatory' monocytes and 'non-classical' monocytes.
In mice, 'classical' monocytes are referred to as $CD11b^{+}$ $Ly6C^{high}$ $CCR2^{+}$ CX_3CR1^{mid} $CD62L^{+}$ and $CD43^{low}$, whereas 'non classical' monocytes can be identified as $CD11b^{+}$ $Ly6C^{low}$ $CCR2^{-}$ CX_3CR1^{high} $CD62L^{-}$ and $CD43^{high}$ [39,40]. In humans, these cells are referred to as $CD14^{high}$ $CD16^{-}$ ('classical') and $CD14^{low}$ $CD16^{+}$ ('non-classical')[41].

1.3.2 Functions of Monocytes and Monocyte Derived Macrophages

Classical Monocytes:
Classical monocytes circulate in the blood during homeostasis but can be quickly recruited upon inflammatory stimuli to a site of injury or inflammation and initiate an inflammatory reaction necessary to clear pathogens or repair injury. After extravasation from the bloodstream through the vessel wall, monocytes enter the tissue and differentiate into mononuclear phagocytes, mostly macrophages but also DCs.

Upon differentiation, monocyte-derived macrophages start upregulating genes necessary for antigen processing and phagocytosis, as well as T-cell co-stimulation. Depending on the tissue that monocytes infiltrate they can additionally upregulate genes needed for specific tissue functions a macrophage has in that tissue[14,20,42].

CCR2 and its ligands CCL2 and CCL7 (MCP3) are the major chemokines involved in monocyte recruitment to tissues. CCR2 is only expressed on a limited number of cell types, while, in contrast, all cells can express CCL2 upon stimulation by inflammatory signals. Although it is known that CCL2 expression is induced during inflammation, its exact mechanism of action remains unknown. One model suggests that CCL2 binds to glycosaminoglycans expressed in tissues to establish a gradient that guides monocytes to the site of inflammation or injury[43,44]. Once monocytes are attracted they have to 'squeeze' through the vessel wall (diapedesis) to access the tissue. In order to do so, monocytes adhere to the vessel wall and traffic through the endothelium. Monocytes will slow down in the bloodstream and start rolling along the vessel wall with the help of integrins (VLA4, LFA1, VCAM1) and selectins (L-selectin, P-selectin, E-selectin) as the major adhesion molecules involved, eventually coming to a stop by binding to chemokines (CCL5, CXCL4 and CXCL5). The cells will then be able to transmigrate through the endothelial vessel wall in an ICAM1-, MAC1-dependent manner and enter the tissue[42,45,46].

Once in the tissue, monocytes mediate antimicrobial actions. One of the best-studied infections is caused by *Listeria monocytogenes*. Upon infection with this Gram-positive bacterium, monocytes are recruited to the liver and the spleen, the two major sites of infection, in a CCR2-dependent manner and differentiate into macrophages that start producing TNF (tumor necrosis factor) and iNOS (inducible nitric oxide synthase), critical in fighting the infection[47,48].

During later stages of the disease, *Listeria* selectively targets and kills Kupffer cells in the liver, which are then replaced by recruited monocyte-derived macrophages[49].

It is now established that tissue macrophage populations are maintained without input from monocytes during homeostatic conditions and that monocytes can be recruited to tissues and become monocyte-derived macrophages upon infections. But exceptions to this rule exist as in certain tissues monocytes contribute to the pool of tissue macrophages, most notably in the intestine. Classical monocytes ($Ly6C^{hi}$) continuously replace the macrophage populations in the intestine, significantly in the lamina propria[9,10,11]. This might be due to macrophage exposure to the microbiota in the intestines, supported by the finding that embryonic macrophages in the intestine are replaced by monocyte-derived macrophages during the first couple of weeks after birth, at the same time when bacteria start colonizing the gut[50].

Other macrophage pools that are slowly replaced by circulating monocytes are found in the testicles[51], the dermis[52], the choroid plexus of the brain[53], the peritoneal cavity[54] and the exocrine pancreas[55]. It is so far unclear if this replacement is triggered by the constant loss of macrophages or is due to that a niche is opened up for monocytes to occupy.

Non-classical monocytes:
The functions of non-classical monocytes are still under investigation but it has been shown that they actively patrol blood vessels for damage, and repair any damage they encounter. In addition it is thought that due to their location, non-classical monocytes might be the first monocytes to enter tissues upon inflammatory stimuli and are also capable of differentiating into macrophages[56,57]. $Ly6C^{lo}$ monocytes are generated from $Ly6C^{hi}$ monocytes in the circulation through a $Ly6C^{hi}$ intermediate which is dependent on the transcription factor C/EBPβ [10,14,58].

1.4 MICROGLIA

Microglia, the macrophages of the brain and spinal cord, are the only resident hematopoietic mesodermal-derived cell type in the central nervous system parenchyma[59]. Even though microglia were already visualized in 1880 by Franz Nissl, who developed the Nissl staining for different neural cells including microglia, it took until 1919 for them to be fully recognized as being ‘microglia’ by Pío del Río Hortega, who is now considered the ‘father of microglia’ due to his research on microglia responses during brain injury[60]. Microglia account for 10-15% of all cells in the brain and are crucial in maintaining homeostasis in both the brain and spinal cord[61]. Microglia constantly monitor the CNS for plaques, damaged neurons or synapses and pathogens[62]. They are among the most sensitive cells in an organism due to unique potassium channels that respond to the smallest changes in extracellular potassium that leads to a reaction of the microglial cell[63].

1.4.1 Development of Microglia

For many years it was believed that adult microglia are established through embryonic and perinatal hematopoietic waves[64,65]. In 2010, Ginhoux *et al*.[8] provided proof that microglia develop from primitive myeloid progenitors that arise before embryonic day 8 (E8). The first evidence was gained by reconstituting sub-lethally irradiated C57BL/6 $CD45.2^+$ newborn mice with hematopoietic cells isolated from $CD45.1^+$ congenic mice. Approximately 30% of circulating leukocytes were of donor origin after 3 months, whereas 95% of microglia were still $CD45.2^+$, suggesting that the microglial pool is not replenished by circulating leukocytes but is of host origin[8]. This is in contrast to previous reports in rats where monocytes were reported to differentiate into amoeboid microglia in the corpus callosum[66] or upon brain injury[67].

Previous to this 2010 study chimeric animals were used to study the origin of microglia. Different results were reported, some in favor of a hematopoietic precursor cell[60,68] and some against[69,70]. Ginhoux *et al.* confirmed both scenarios using bone marrow chimeras as well as parabiotic animals. In order to transplant new bone marrow mice need to be irradiated, which damages the blood brain barrier (BBB), making it possible for bone marrow cells to pass the BBB and enter the brain. This leads to differentiation of hematopoietic precursors into microglia upon infiltration of the CNS[69,70,71]. Conversely, in parabiotic mice, which share a blood circulation, the microglial pool was only replenished to a minimal extent by hematopoietic precursor cells, whereas other tissue macrophages were replaced by up to 30%, even after 12 months of parabiosis[8].

Using a fate mapping approach, Ginhoux *et al*. further studied the origin of microglia during development. Using an inducible runt-related transcription factor 1 (*Runx1*) CreER system they were able to label *Runx1*-expressing cells at E7.0, which is prior to definitive hematopoiesis in the mouse embryo. Using this system they could convincingly show that *Runx1*-expressing yolk-sac-derived macrophages persist into adulthood as microglia[6,8].

1.4.2 Functions of Microglia in Homeostasis and Disease

Microglia are commonly referred to as ‘brain macrophages’ due to many similar functions to other tissue macrophages, but they are unique when it comes to their origin, their homeostatic phenotype and their tight regulation by the CNS microenvironment. Microglia are not only important for the elimination of pathogens and resolving of inflammation and injury, they also have a major role in regulating brain development and maintaining neuronal networks[72].

Microglia continuously monitor the brain during homeostasis by extending and protruding their branches and processes. These ‘resting’ or ‘ramified’ microglia cover huge areas and make contact with neurons, astrocytes and blood vessels in order to ensure functionality of synapses and that there are no pathogens or dead cells that can threaten CNS homeostasis[73,74,75]. If microglia encounter an injury, inflammatory event or pathogens, they become activated (amoeboid morphology).

Their cell bodies swell and their processes shorten, making them highly activated pro-inflammatory cells capable of very efficient phagocytosis and elimination of intruders[76]. Just like macrophages, microglia express different pattern recognition receptors (PRR) that sense pathogens via pathogen-associated molecular patterns (PAMPs) and danger via danger-associated molecular patterns (DAMPs), for example Toll-like receptors (TLR 1/2 and TLR4), NOD-like receptors (NLRP3 inflammosome) and scavenger receptors that enable them to perform phagocytosis e.g. CD36, MARCO and SR1[77,78]. In addition, microglia express various chemokine receptors, most prominently CX3CR1 and CX3CR4, as well as integrins (CD11b, CD11c) that help control microglial positioning and movement in the CNS[79,80]. Microglial activation is regulated by immune receptors, the best studied being TREM2 (triggering receptor expressed on myeloid cells 2) and CD33[81,82] and the inhibitory receptors SIRPA as well as CD200R1[83,84].

Microglia aid in the formation of neuronal networks during development by guiding other glial cells and neurons[85]. In adults, microglia comprise a major part of the neurogenic niches in the subventricular zone (SVZ) and the subgranular zones (SGZ) in the dentate gyrus, which produce neurons that accommodate the olfactory bulb and hippocampus. Microglia are attracted to these neurogenic niches by factors released from apopototic neurons (f.ex. ATP) and CXCL12[86]. In adults, microglia are crucial for shaping synaptic spines by phagocytosing retired dendritic spines, a process called 'synaptic stripping'[87,88]. Synapse plasticity and strength are regulated, in part, by pro-inflammatory factors released by microglia as well as CX3CR1-CX3CL1 interactions. Mice lacking CX3CR1-CX3CL1 have impaired connectivity and afferent synaptic inputs in the hippocampus[89].

Taken together, microglia serve on the one hand as the macrophages of the brain, combating pathogens and phagocytosing dead cells and debris, and on the other hand they aid brain development by helping form neuronal networks and playing a crucial part in the adult in synaptic pruning – this unique homeostatic functions distinguish microglia from other tissue macrophages in the body. In addition, microglia display a unique transcriptional profile with many genes that do not overlap with other resident tissue macrophage genes.

1.4.3 Experimental Depletion Models

Monocyte/Macrophage Depletion Models:
Depletion of macrophages and monocytes is nowadays a commonly used method to study niche signals defining tissue macrophages or to elucidate the role of macrophages in various diseases. The first macrophage depletion technique was introduced in the 1980s by van Roijen, who used clodronate-encapsulated liposomes to deplete circulating monocytes[90]. A more targeted approach to experimentally deplete Ly6C^{hi} monocytes is through using antibodies against CCR2, or *CCR2*$^{-/-}$ mice in which Ly6C^{hi} monocytes are prohibited from leaving the bone marrow and entering the circulation[91,92]. The generation of the CD11b-DTR mouse[93], in which upon diphtheria toxin (DT) administration macrophages can be efficiently depleted at chosen time points, made it possible to study macrophage ablation in even more detail.

In these mice the transgenic expression of the human DT receptor (DTR) confers sensitivity to DT and permits ablation *in vivo* when DT is injected, due to the fact that the mouse DT receptor only binds DT poorly compared to the human counterpart[93,94].

Microglia Depletion Models:
Clodronate liposomes do not ablate the microglial pool because they do not cross the BBB. In order to overcome this obstacle to study microglia depletion, various groups injected clodronate liposomes intracerebroventricularly (i.c.v), leading to efficient depletion of the microglial pool[95,96,97]. A major step forward in the field of microglia depletion was the development of the CD11b-HSVTK mouse, which expresses the herpes-simplex virus encoded suicide-gene thymidine kinase under the CD11b-promoter[98]. About 95% of microglia are depleted upon gancicolvir injection using an osmotic pump in this system[99]. However, ganciclovir treatment becomes toxic after prolonged administration of 4 weeks. Upon withdrawal of ganciclovir, microglia are entirely exchanged by peripheral myeloid cells[100,101].

To allow a more specific depletion of microglia, *Cx3cr1*^{Cre} mice were developed. When bred with *Rosa26*^{DTR} mice, microglia are depleted without affecting CX3CR1^{+} bone marrow cells[102,103,104,105] upon Tamoxifen and diphtheria toxin administration. Since microglia require CSF1R for their development (*CSF1R*$^{-/-}$ mice are born without microglia[106]), inhibiting this receptor pharmacologically leads to 99% microglia depletion within 21 days, making it a faster approach for efficient microglial depletion[8,106,107]; however, peripheral macrophages are also depleted, making the genetic model more favorable for specific targeting of microglia.

1.5 EOSINOPHILS

Paul Ehrlich was the first to describe eosinophils in 1879 after noticing their potential to be stained by acidophilic dyes, hence the term ‘acidophiles’. Even though eosinophils were described a long time ago, their functions still remain elusive to a large extent[108]. Eosinophils differ in morphology, cell-surface receptor expression and intracellular content among species, but are conserved in all vertebrates[109]. In human blood, mature eosinophils only circulate in small numbers (400 cells per mm^3 blood) during homeostasis, while their numbers can increase rapidly upon infection when eosinophils leave the bone marrow upon certain stimulation[110]. Eosinophils have mostly been studied in parasitic infections and allergic reactions such as asthma[111,112,113], but it is becoming clear that they have implications in many more diseases, including cancer[114,115,116,117].

1.5.1 Development of Eosinophils

Eosinophils develop in the bone marrow from a hematopoietic precursor, the GATA-1^{+} common myeloid progenitor (CMP). After progressing through the pre-granulocyte macrophage progenitor stage (Pre-GMP) they further differentiate into GATA-1^{+} granulocyte and macrophage progenitors (GMP, also known as eosinophil/mast cell progenitors EoMP).

IL-33 regulates further development and expansion into eosinophil precursors as well as inducing a currently unidentified cell that starts producing IL-5 that is required for final eosinophil maturation[118]. Three transcription factor families, GATA-1, PU.1 and C/EBP, define eosinophil lineage specification. Mice lacking the high affinity GATA-1 binding site do not have eosinophils, making it the most important transcription factor involved in eosinophil development[119]. Even though other cell lineages are also GATA-1$^+$ (mast cells, megakaryocytes, erythroid cells), it appears that GATA-1 has specific functions in eosinophils, very likely mediated by a palindromic GATA site. This specific GATA site is present in the downstream GATA-1 promoter region, as well as regulatory regions of eosinophil-specific genes (*CCR3*, *MBP*, *IL-5Ra*) and it is responsible for eosinophil specific gene expression[120,121,122].

IL-5 is necessary for selective differentiation of eosinophils, their release from the bone marrow and their maturation[123]. Over-expression of IL-5 in transgenic mouse models leads to extensive eosinophilia[124,125,126], whereas deletion of the IL-5 gene leads to a significant reduction of eosinophil numbers in the blood and lungs, even after allergen challenge[127,128]. In humans, disease-associated eosinophilia is also regulated to a major extent by IL-5 and treating these patients with humanized anti-IL-5 antibodies can drastically reduce eosinophil numbers in the blood and to a certain extent in the lungs of patients[129, 130,131].

1.5.2 Functions of Eosinophils in Homeostasis and Disease

Eosinophils were for a long time considered as end-stage effector cells involved in defense against pathogens[132]. It is now known that eosinophils also have many more functions during development and other biological processes, ranging from mammary gland development[133], viral infections[134], estrus cycling[135], allergic responses, neoplasia[136] to involvement in organ transplantation[137].

Eosinophils in Homeostasis:
Eosinophils are found abundantly in the female reproductive tract, with numbers increasing during the estrus cycle. IL-5 attracts eosinophils to the uterus, but it was shown that some eosinophils are IL-5 independent since mice lacking IL-5 have a residual population of eosinophils in the uterine stroma[135]. Ovarian hormones influence eosinophil chemoattractants (eotaxin-1, RANTES) leading to influx of eosinophils. Mice lacking eotaxin-1 do not have uterine eosinophils and have a two-week later onset of estrus as well as a delayed age of parturition[138,139].
Eosinophils are also implicated in postnatal mammary gland development. Eotaxin-1 mRNA levels are increased during mammary gland formation, correlating with eosinophil influx into the tissue. Mice lacking eotaxin-1 have a deficiency in terminal end bud formation and reduced branching complexity of the ductal tree[140].
Recruitment of eosinophils to the cortico-medullary region of the thymus during the neonatal period is also regulated by eotaxin-1. A second wave of eosinophils, expressing high levels of MHCII, infiltrates the thymus at a later stage, coinciding with the commencement of thymic involution[141].

Eosinophils in Immune Regulation:
Eosinophils perform many functions in immune regulation such as antigen presentation[142,143] and release of cytokines and lipid mediators to mediate inflammatory responses[112,144]. Eosinophils are capable of processing and presenting antigens from various pathogens, including microbes, parasites, and viruses[144], promoting proliferation and polarization of $CD4^+$ T-cells. Eosinophil antigen presentation capabilities have mostly been studied *in vitro*, with discrepancies in the results, most likely due to different eosinophil isolation techniques used[145]. Eosinophils secrete cytokines (IL-2, IL-4, IL-6, IL-10, IL-12) that activate T-cells and mount an effective immune response to invaders, again demonstrating a tight relationship between these two cell types[146]. In addition, eosinophils impact secretion of IL-4, IL-5 and IL-13 by T-cells, cytokines necessary for eosinophil maturation and activation[142,143,147].

Eosinophils and Cancer:
Many studies in various types of solid cancers (colon cancer [148,149], squamous cell carcinomas[150], bladder cancer[151]) have revealed that tumor-associated tissue eosinophilia (TATE) has a positive effect on patient survival and outcome. In contrast, TATE in Hodgkin lymphoma patients seems to be an indicator for poor prognosis[152].

TATE is mostly observed in necrotic areas of the tumor and eosinophil migration is induced by necrotic cells both *in vivo* and *in vitro*[153,154]. Necrotic cells release eosinophil-derived high-mobility group box 1 (HMGB1) that binds to RAGE on eosinophils and triggers eosinophil degranulation[155].
Tumor cells also produce other cytokines (IL-5, IL-3) and chemokines (eotaxin-1, CCL17) that induce eosinophil maturation and migration to the tumor site[156].
Cytotoxic anti-tumor responses are proposed to act through degranulation of eosinophil granules, but this is still debated and not well understood. One factor strengthening this hypothesis is observations of granule proteins in the tumor vicinity[157]. A recent study determined that eosinophils help to reject cancer by normalizing blood vessels and enhancing infiltration of tumor-specific $CD8^+$ T-cells[114]. Eosinophils may also alter the tumor microenvironment to become less immunosuppressive by changing the polarization states of macrophages to a more pro-inflammatory type, leading to tumor eradication and longer survival[114]. IL-2 immunotherapy is a common treatment for melanoma and renal cell carcinomas. Part of the antitumoral effect of systemic IL-2 therapy is thought to be due to degranulation of eosinophils within tumors[158,159].

1.6 MALIGNANT GLIOMA

Primary brain tumors account for about 2% of cancers in the western world and can generally be classified as glioma (astrocytoma, oligodendroglioma, ependyomas) or non-glioma (menigiomas, pituitary tumors, medulloblastoma). Malignant gliomas account for about 30% of all brain tumors and are the most common subtype of primary brain tumors, characterized by their highly aggressive, invasive and neurological destructive nature[160].

Malignant gliomas are considered to be among the deadliest human cancers with glioblastoma multiforme (GBM) being the most frequent and aggressive form[161]. This cancer, as with many other human cancers, involves a variety of cellular processes and genetic and epigenetic DNA modifications needed to initiate (formation), promote (proliferation) and progress (migration)[162,163].
Despite recent advances in glioma biology the median survival has changed little during the last decade and only ranges from 12-14.5 months[164]. Up until now the gold standard of therapy has been maximum surgical resection of the tumor mass followed by radiotherapy and Temozolomide chemotherapy according to the FDA.

The first brain tumor surgery was performed in 1884 to much acclaim by Rickman Godlee, but soon after the initial enthusiasm faded because it became clear that survival was only slightly prolonged using this approach. Thirty years later Harvey Cushing and Percival Bailey suggested the first grading scheme for malignant gliomas based on morphological appearance. They described the diffuse nature of gliomas, indicating cure of the disease through surgery alone was quite impossible. To date, only the invention of chemotherapeutic agents that can cross the blood brain barrier (in the early 20th century) has increased the median survival of glioma patients by a couple of months from a prior maximum of 12 to a maximum survival of 15 months[165].

Since then great progress has been made in the field of immunotherapy and other cancer treatments for different kinds of cancers such as breast, lung and prostate cancers, as well as for melanomas and leukemias. However, it is stunning how inefficient these approaches are in the treatment of malignant gliomas[160].

1.6.1 Grading of Gliomas

The most commonly used scheme for grading and classification of gliomas was published by the World Health Organization (WHO). Gliomas are thus classified according to their line of differentiation, being either astrocytic, oligodendroglial or ependymal cells. Astrocytic tumors are further classified into pilocytic astrocytoma, astrocytoma, anaplastic astrocytoma and glioblastoma. Furthermore, gliomas are graded into classes I to IV depending on their degree of malignancy[166,167]. This grading is related to histological features of the tumor such as high cellularity, cellular pleomorphism, mitotic activity, microvascular proliferation and necrosis.

Table 1: Grading scheme for CNS tumors

Grade I	Tumors are benign and can be surgically cured if resection is possible.
Grade II	Slow growing tumors that can be malignant or non-malignant and follow long clinical courses. Can recur as higher grade tumors.
Grade III	Malignant tumors, leading to death within a couple of years after diagnosis.
Grade IV	Highly malignant tumors, usually unmanageable by surgery, radiotherapy and chemotherapy and lethal within 12-15 months.

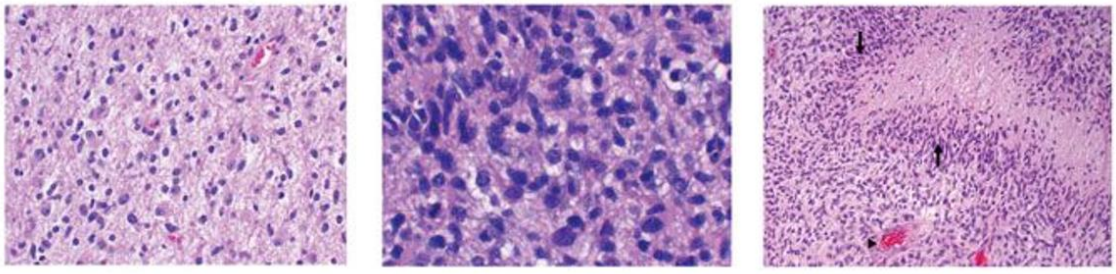

Figure 3: WHO classification for glioma. Hallmarks of GBM: microvascular proliferation *(black arrowhead)*; pseudopalisading necrosis *(black arrows)*.

In 2016 the new WHO classification of CNS tumors was introduced, using molecular parameters in addition to histology to describe the different tumor entities. GBM is now classified as being either IDH-wildtype (about 90% of cases, corresponding to primary GBM), IDH-mutant (corresponding to secondary GBM) or GBM NOS for which a full characterization of the IDH mutation status cannot be performed[168,171].

1.6.2 Glioblastoma multiforme

About 3 in 100,000 people are newly diagnosed with GBM annually, accounting for more than 51% of all gliomas. More than 90% of these cases are primary GBMs that arise from normal glial cells through oncogenesis, typically in older patients without any prior signs of disease (>62 years). The other 10% are secondary gliomas that originate from lower grade tumors (mostly astrocytomas). These secondary gliomas expand distinctly more slowly than the primary GBMs and are common in younger patients (<45 years). Secondary GBM transforms from low to high-grade tumors within 4-5 years, regardless of prior therapy[160,169,170]. Even though these two subtypes of GBM have different genetic profiles and are therefore considered to be two distinct clinical entities, no distinctions are made in terms of treatment. However, in terms of prognosis, primary gliomas are considered worse than are secondary gliomas.

Etiology of GBM:

Malignant gliomas or GBM are thought to derive from neuroectodermal stem cells or tumor progenitor cells[168]. They are spontaneously occurring tumors associated with a deregulated G1/S checkpoint in the cell cycle leading to further mutations in glioma cells (e.g. loss of heterozygosity on chromosome 10q, amplification of EGFR, AKT3, FGFR2 and mutations in PTEN, TP53 or NF1)[171]. In rare cases GBM can also occur due to a genetic predisposition; for example individuals with tuberous sclerosis[172], Turcot syndrome[173], multiple endocrine neoplasia type IIA[174] or neurofibromatosis type I[175] were reported to be more prone to developing GBM.

Apart from ionizing radiation, certain chemicals, common mutations and pre-existing genetic diseases as etiologic factors, the human cytomegalovirus (HCMV) is also believed to cause GBM. This virus encodes different proteins involved in mutagenesis, apoptosis, inflammation, angiogenesis and mitogenesis, leading to a dysregulation in key signaling pathways such as PDGFR, Akt and STAT3[176,177]. Single nucleotide polymorphisms (SNP) in IL-4Rα (interleukin 4 receptor) and IL-13 genes were also identified as risk factors for the development of GBM, and this suggests that tissue inflammation plays a role in the process of GBM development[178].

Clinical and Morphological Features of GBM:

GBM is a tumor of the central nervous system (CNS), most often located in the hemispheres or subtentorial in the brain stem and cerebellum[179,180]. Due to its infiltrative nature it is difficult to distinguish the tumor mass from normal brain tissue, making complete surgical resection impossible. GBM barely metastasizes to other organs, most likely due to the isolated location of the brain and the barrier created by cerebral meninges but also the aggressiveness and therefore short course of disease might be a reason [181].

Clinical symptoms of GBM are very much dependent on the age of the patient, location and the size of the tumor and how fast it grows. Clinical signs of the disease include severe headaches, dizziness, seizures, increased intracranial pressure and neurological deficits or changes in the patients' mental status[182].

GBM is usually diagnosed using gadolinium-enhanced MRI examination whereby it typically presents with irregular contours and strong contrast enhancement around a darker necrotic area[183] and is morphologically profoundly different from normal brain tissue. The cells are small polygonal to spindle shaped with indistinct cellular borders and oval or elongated nuclei with multiple nucleoli. Binuclear and multinucleated cells are also present, as are lymphocytes, neutrophils, macrophages and necrotic cells[184]. The most prominent feature of GBM is necrotic foci, either in the central area of the tumor or small irregularly shaped necrotic foci surrounded by pseudopalisading areas.

The treatment of GBM comprises maximum surgical resection of the tumor mass, radiotherapy and chemotherapy. Due to the complex nature of the tumor, these treatments only minimally increase the patients' life expectancies.

GBM tumor cells have a highly infiltrative nature into the surrounding brain tissue and easily evade surgical intervention as well as radio- and chemotherapeutic interventions. They do so by down-regulating certain tumor suppressor proteins, production of immunosuppressive cytokines and up-regulation of DNA repair enzymes[185]. Chronic inflammation and the recruitment of regulatory T-cells as well as myeloid-derived suppressor cells effectively interfere with innate and adaptive anti-tumor immune responses[186].

1.7 MALIGNANT MELANOMA

Melanoma is the deadliest form of skin cancer and incidences are rising despite the fact that the major cause of melanoma, exposure to the sun and UV radiation, is well described[187,188]. Development of melanoma is not only influenced by the time of exposure, but also the patterns of exposure seem to be important[187]. Even though preventive measures are available, most importantly sun protection, only a limited number of people use them. Melanoma is a highly treatable cancer, but once it metastasizes prognosis is poor[189].

Etiology and Therapy of Malignant Melanoma:
Melanoma arises due to genetic mutations in the pigment-producing cells, melanocytes, of the skin, the eye, inner ear and leptomeninges. Melanocytes are the only cell type in the body producing melanin pigments from tyrosine and cysteine precursors[190], a complex biochemical mechanism. Due to its complexity, melanocyte intracellular systems, compartmentalization, detoxification and repair are prone to damage[190].

Chemotherapy was the first treatment available for malignant melanoma, and even though treatment regimens have been improved and different combinations have been tested, overall survival was not affected[191,192]. Although better treatments are on the market, chemotherapeutics are still used for palliative treatment of refractive, progressive and relapsed melanomas[193].

Immunotherapy is a promising approach to target melanoma due to the fact that melanoma is a highly immunogenic cancer[194]. Already in the 1900s it was noticed that tumors are infiltrated by immune cells and are inflammatory hotspots, but only now is this knowledge used to design effective therapies[195].

Interferons (IFNs) activate and inhibit certain cell types. T-cells, B-cells, natural killer cells and DCs are commonly activated by IFNs, whereas regulatory T-cells and MDSCs are inhibited. IFNs can interfere with viral replication but also play an inherent role in immunomodulatory, antiangiogenic, antiproliferative and antitumor activities[196]. Treatment with IFN-α has an immunomodulatory antitumor effect by inhibiting the proliferation of melanoma cells due to a stimulatory effect on MHCI expression on immune cells and melanoma cells as well as inducing apoptosis upon certain doses[197,198].

CTLA-4 and PD-1/PD-L1 are the newest generation of immunotherapeutics for the treatment of various cancers, including malignant melanoma. They belong to the class of checkpoint inhibitors that block T-cell activation to induce immune tolerance[199,200]. Blocking these checkpoints with antibodies interferes with the inhibitory effects, enhancing the production of pro-inflammatory T-cell cytokine production and T-cell expansion[201], which infiltrate the tumor and attack tumor cells. Many adverse effects have been reported, including dermatitis, colitis, drug-related hepatitis, endocrinopathies and neuritis[202]. Several clinical trials are currently investigating combination therapies using checkpoint inhibitors together with chemotherapy, radiation therapy or other immunotherapies[189].

Other treatment strategies comprise for example adoptive T-cell therapy (ACT), where patients are infused with melanoma specific T-cells (ideally isolated from the patient themselves), but the generation of these cells is complicated, expensive and requires a lot of time[203]. Targeted therapies are available, targeting and inhibiting the most commonly mutated genes in melanomas, *BRAF*, *MEK*, *VEGF* and *PI3K-AKT-mTOR* pathway[189].

1.8 TUMOR MICROENVIRONMENT

The close interplay between tumors and their microenvironments was already suggested in 1889 by Stephen Paget. He postulated in his 'seed-and-soil' theory that metastases of one type of cancer (seed) is more likely to metastasize to sites similar to the original and secondary tumor sites (soil) (Paget 1889). Nowadays it is known that the tumor microenvironment is the (cellular) environment in which the tumor exists. This includes lymphocytes and inflammatory cells but also blood vessels, immune cells, fibroblasts, as well as signaling molecules (for example cytokines and chemokines) and extracellular matrix proteins. The tumor interacts with and influences its microenvironment by releasing extracellular signals to promote tumor growth, angiogenesis and inducing peripheral immune tolerance to escape immune surveillance[186,204]. Immunosuppressive cytokines such as prostaglandin E2, TGF-β and IL-10, among others, are highly expressed in GBM and tumor infiltrating T-cells consist of an enriched population of $CD4^+/CD25^+/FoxP3^+$ regulatory T-cells (Tregs)[205], making the tumor microenvironment even more immunosuppressive. Additionally, various tumor stem cells have also been reported to be immunosuppressive[206].

2 METHODS

2.1 CRE-LOX SYSTEM

To better study specific genes and their functions in specific cell types, tools have been developed that allow the introduction of mutations into the mouse genome at a certain time point, in certain tissues and even in certain cell types[207]. The Cre/lox site-specific recombination technique is the most commonly used tool to generate mouse mutants and allows control of gene activity in almost any mouse tissue. This allows the study of gene function in more detail and helps develop more sophisticated animal models of human disease[208]. The Cre/lox system is based on the use of a site-specific recombinase Cre (cyclization recombination), which catalyzes recombination between two recognition sites, loxP (locus of crossing over of bacteriophage P1)[209]. To delete genes two loxP sites are introduced that flank an essential exon of the gene of interest by homologous recombination in embryonic stem cells. Cre then excises the targeted exon from the chromosome, generating a null allele in all cells that express Cre. Cre can be delivered by crossing mice carrying the loxP sites with mice carrying the Cre of interest. To achieve cell-specific or tissue-specific gene deletion, a Cre-recombinase that is under the control of a specific promoter is used.

A major breakthrough in the Cre/lox system was the development of ligand-dependent Cre recombinases (CreER) that can be activated by administration of tamoxifen to the animal. Using this system, Cre activity can be turned on at any time [210,211]. I used this system in **Paper I**[214] and **Paper II**[215], studying microglia depletion and deletion of *Tgfβr2* in distinct cell subsets.

2.2 CX3CR1-DTA MICROGLIA DEPLETION MODEL

Depletion of microglia is commonly used to study the role of microglia in homeostasis and disease. As reviewed in chapter 1.4.3, many different techniques have been developed to target microglia, but a major breakthrough was the development of *Cx3cr1*^{CreER} mice[102,103]. Specific depletion of microglia in these mice is achieved when crossing them with *R26*^{DTR} mice, which have DTR inserted into the ubiquitously expressed Rosa26 locus[212]. Even though microglia are efficiently depleted to up to 80% in these mice, rapid proliferation of microglia compensates for the loss of cells[104]. To overcome this we crossed *Cx3cr1*^{CreER} mice to *R26*^{DTA} mice [214]. Tamoxifen administration in these mice results in almost complete depletion of the microglial niche (>95%), yet also in this model repopulation occurs through a combination of CNS-resident microglia repopulation and infiltration of Ly6C^{hi} monocytes, distinguishable by their expression of F4/80. This model was used to study depletion and repopulation in **Paper I**[214] and the role of *Tgfβr2* in repopulating cells in **Paper II**[215].

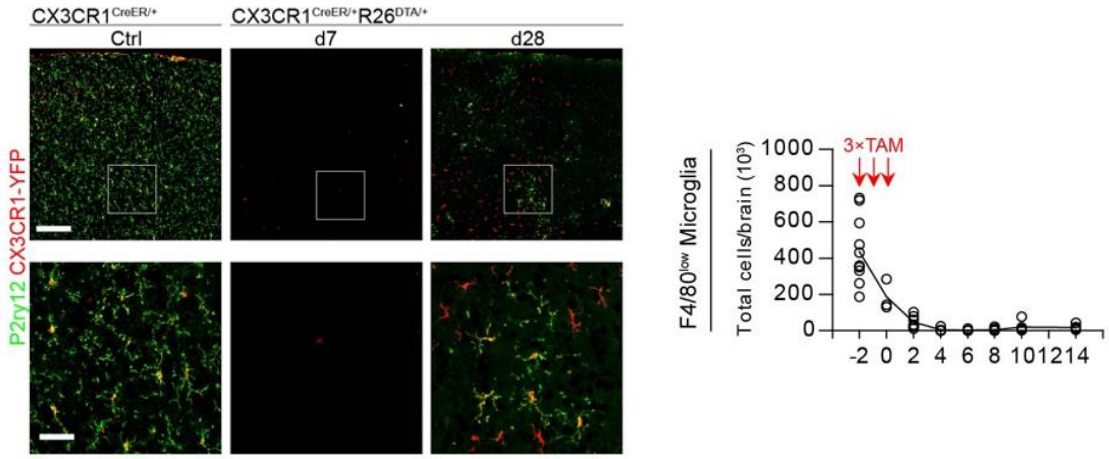

Figure 4: Efficient depletion of microglia followed by repopulation using the *Cx3cr1*^{CreER}*-R26*^{DTA} mouse model. P2ry12 *(green, microglia specific)*, CX3CR1-YFP *(red)*, overlap *(yellow)*.

2.3 GL261 GLIOBLASTOMA MODEL

GL261 is a syngeneic mouse model in C57BL/6 mice. Since these mice do not require a deficient immune system, the GL261 model is thought to mimic GBM growth and immune responses of human GBM more closely than other mouse models. The model was developed by Seligman and Shear in 1939, who implanted 20-methylcholantrene pellets into the brain parenchyma of mice[216,217]. GL261 tumors are characterized by their poorly differentiated cells with a morphology similar to human GBM cells[218,219] and their infiltrating and invasive characteristics into normal brain tissue. Individual cells can be found several millimeters away from the tumor margin and show several of the 'secondary structures of Scherer': perineuronal satellitosis, perivascular satellitosis, subpial spread and incasion along the white matter tracts[219,220]. They also show areas of pseudo-palisading necrosis, similar to human GBM and express human GBM markers such as GFAP and S100[216]. The GL261 mouse model also shares some genetic mutations with human GBM, for example point mutations in the *K-Ras* and *p53* genes[221,222] as well as increased activation of the PI3K pathway[223].
Due to its resemblance to the human disease, not only in cellular composition and invasiveness but also in tumor aggressiveness, the GL261 model is a good choice to study immune components of GBM and potential immunotherapeutic approaches. I used the GL261 model in **Paper III** to study the immune cell composition of the tumor and target different cell types with depletion techniques.

2.4 B16 MELANOMA MODEL

As for GBM, also in melanoma the advantage of using a syngeneic transplantation model is that the animals are immunocompetent, which allows melanoma cells to interact with T-cells and B-cells naturally, as they do in the human melanoma microenvironment[224]. The B16 melanoma cell line is the most widely used cell line, especially in the study of immune-tumor interactions and immune therapeutics. The cell line was chemically induced to produce spontaneous tumors.

Two main clones are commonly used, B16F1, which has low metastatic potential and is used to study primary tumors and B16F10, which has a high metastatic potential to distant visceral organs[225,226]. The B16 model differs vastly from the human disease in genetic mutations, most notably for *Braf*. While the human disease has at least 60% of tumors mutated in *BRAF*, the mouse model only exhibits minor mutations in this gene[227]. *PTEN*, another commonly mutated gene in human disease, is not usually mutated in B16 cells[227]. Another factor that has to be considered when using this melanoma model is that B16 cells proliferate fast, making long-term studies virtually impossible due to ethical considerations[228]. As with every other animal model there is skepticism towards the validity of the model when comparing to the human situation, but for basic studies the B16 syngeneic mouse model is suitable[229]. I used the B16 melanoma mouse model to study the effect of siRNA delivered by nanoparticles in **Paper IV**.

3 RESULTS AND DISCUSSION

3.1 PAPER I: COMPETITIVE REPOPULATION OF AN EMPTY MICROGLIAL NICHE GIVES RISE TO FUNCTIONALLY DISTINCT SUBSETS OF MICROGLIA-LIKE CELLS

Aim:
To develop an efficient long-term microglia depletion model in order to study niche competition.

Background:
Circulating monocytes can compete for any tissue macrophage niche and become long-lived replacements that are phenotypically indistinguishable from their embryonic counterparts under specific conditions. Factors regulating this process are not well understood.

Hypothesis:
Microglia can be efficiently depleted followed by repopulation by different sources without affecting health and cognitive functions in mice.

Results:
Cx3cr1^{CreER}-*R26*^{DTA} mice are depleted of microglia by >95% after Tamoxifen treatment. Within weeks the microglial niche is repopulated by a combination of local proliferation of CX3CR1^{+}F4/80^{low} microglia and infiltration of CX3CR1^{+}F4/80^{hi} macrophages. Infiltrating macrophages arise directly from Ly6C^{hi} monocytes entering the brain from the periphery. This colonization by monocytes is independent of blood brain barrier disruption, does not require progenitor cells, is accompanied by vascular activation and regulated by type I interferon.
Ly6C^{hi} monocytes upregulate microglia-specific gene expression and adopt a microglial DNA methylation profile, but still retain a distinct gene expression profile. Our results demonstrate that monocytes can be imprinted by the CNS microenvironment but remain functionally, epigenetically and transcriptionally distinct cells.

In **paper I** we developed a new model for microglia depletion by breeding *Cx3cr1*^{CreER} mice with *R26*^{DTA} mice. By doing so, microglia are efficiently depleted by more than 95% within 7 days upon Tamoxifen administration to the adult offspring. As in all other depletion models, be it pharmaceutically or genetically, the microglia niche becomes repopulated within 14 days after depletion. This does not make our model unique *per se*, but that repopulation occurs not only from the naïve microglia pool through proliferation but in addition through infiltration of Ly6C^{hi} monocytes from the periphery into the brain is indeed novel in our model, and opens up new possibilities to research cell replacement therapies. To date, microglial turnover (in steady state) or repopulation (upon depletion) was only reported to occur either through microglial proliferation[70,104,107,230,231] or through infiltration of peripherally derived myeloid cells[61,66,69,100,232,233], but not to occur simultaneously.

In addition, most studies reporting engraftment of peripheral cells in the brain were performed in models in which damage to the BBB cannot be excluded[69,70,100,104,233], leading to the conclusion that it is an experimental artefact. In our model, however, we prove that the BBB is undisrupted and infiltration by peripheral myeloid cells into the CNS still occurs, which supports previous findings by Cronk *et al.*[232].
In this discussion I try to shed some light on past and recent publications of microglial depletion and repopulation studies, and their differences as well as similarities to our own findings in **Paper I**.

Disruption of the Blood Brain Barrier:
BBB disruption following whole body irradiation is considered as one of the major factors for infiltration of peripheral myeloid cells into the parenchyma. Ajami *et al.* reported in 2007 that local microglia proliferation sustains the CNS microglia population in the adult, whereas infiltration of peripheral bone marrow cells is possible upon lethal irradiation of the mouse[70]. Similarly, in the *Cx3cr1*^{CreER}*-R26*^{DTR} depletion mouse model, Bruttger *et al.* reported that microglia are only repopulated from the periphery after depletion when the mice are lethally irradiated without head protection, and become reconstituted with new bone marrow-derived cells[104]. Both studies attribute this infiltration to damage of the BBB as a result of irradiation. In contrast, a paper published in 2012 very convincingly reported that lethal irradiation using 10Gy does not affect the integrity of the BBB[234], as measured by IgG and albumin content in the brain upon whole body irradiation. Damage to the BBB was reported in earlier studies, but higher doses (20-50Gy) were used in those[235], leading to the conclusion that disruption of the BBB only happens at high doses of irradiation.

In further support of our finding that disruption of the BBB is not necessary for peripheral cells to enter the brain is the report by Cronk *et al.* who showed that chronic depletion of microglia in a *Cx3cr1*^{CreER}*-CSF1R*$^{fl/fl}$ mouse model by feeding Tamoxifen chow also leads to repopulation of the microglial niche by infiltrating cells[232]. However, BBB damage and cell infiltration can indeed be an experimental artifact in the CD11b-HSVTK microglial depletion model, where insertion of an intracerebroventricula cannula is necessary for constant ganciclovir administration[100], but no experimental data was provided to prove disruption of the BBB.

Inflammation in the Brain upon Microglia Depletion:
Increased astrocyte activation and pro-inflammatory cytokine expression in the brain was evident after depletion of microglia in our *Cx3cr1*^{CreER}*-R26*^{DTA} model, which was largely resolved after two weeks. Other studies reported short-term inflammation of the brain following depletion, which is most likely attributable to massive microglial death in the tissue. For example, Bruttger *et al.* reported that inflammatory cytokines are enhanced in their *Cx3cr1*^{CreER}*-R26*^{DTR} model in which 80% of microglia are depleted, but no infiltration of peripheral cells occurs during repopulation[104].

Cronk *et al.* demonstrated that a chronic depletion of 25% of the microglial pool is sufficient to trigger peripheral myeloid cell infiltration[232].

Even though inflammation seems to be a common effect in various different depletion models, inflammation alone is therefore most likely not the reason why bone marrow cells infiltrate the brain and permanently engraft there.

Introduction of Stem Cells into the Circulation:
Ajami *et al.* reported that during homeostasis microglia sustain their pool by local proliferation and that peripheral cells only contribute if the BBB is disrupted and cells are transplanted. By irradiating one partner of a parabiotic pair and not irradiating the other, they demonstrated that irradiation and BBB disruption alone was not sufficient for peripheral cells to contribute to the microglia pool, but that cell transplantation is needed for this to happen[70]. A subsequent report indicated that infiltration of the brain occurs through precursor/stem cells that are released into the blood in increased amounts upon irradiation without passing through a monocyte intermediate. This finding was supported by Bruttger *et al.* who used an anti-CCR2 antibody to demonstrate that infiltration following irradiation is not affected upon depletion of $CCR2^{+}$ monocytes in the circulation[104]. In contrast, we show that $Ly6C^{hi}CCR2^{+}$ monocytes infiltrate the CNS, which further permanently engraft in the CNS using adoptive transfer of purified $Ly6C^{hi}$ $CCR2^{+}$ monocytes in **Paper I**. Our findings were confirmed by Mildner *et al.* who identified $Ly6C^{hi}CCR2^{+}$ monocytes as direct 'precursors' of microglia also by using bone marrow chimeras and adoptive transfer models. Mice lacking $Ly6C^{hi}CCR2^{+}$ monocytes did not exhibit any engraftment into the brain[69]. In further support of monocytes being microglia 'precursors' Cronk *et al*. also reported stable $Ly6C^{hi}CCR2^{+}$ monocyte engraftment upon adoptive transfer[232] in parabiotic mice.

Impairment of Microglia Proliferation:
Even though local microglia proliferation partly reconstitutes the empty microglia niche in our model it is not efficient enough to fill the entire niche, and hence infiltrating cells are recruited. Our conclusion is that the proliferative ability of microglia is compromised in our model. In support of this are the findings of the chronic microglia depletion model used by Cronk *et al.*. Conversely, in clear opposition to these findings, CSF1R inhibitor-treated mice, in which 99% of microglia are depleted, do not exhibit any signs of peripheral input, but instead repopulate the niche through extensive proliferation of the surviving microglia[231,236]. CSF1R treatment very likely also inhibits monocytes from repopulating the brain. Also in contrast to our findings, $Cx3Cr1^{CreER}$-$R26^{DTR}$ recover by microglial numbers within days through hyperproliferation[131].

In conclusion, factors influencing engraftment of peripheral cells in the brain and local microglial proliferation need to be studied further in order to draw conclusions and use these models for cell replacement therapies. In addition to establishing a new model of microglial depletion in which repopulation of the niche occurs without BBB disruption and without input from stem cells, we were further able to identify microglia and 'bone marrow-derived' microglia-specific genes, making it possible to distinguish them.

Cronk *et al.* also defined core signatures of brain engrafting macrophages (beMΘ-50) and microglia (Mg-52)[232], largely overlapping with our own dataset, which strongly supports our data. Two other recent publications shed additional light on this issue. In support of our data, graft-derived macrophages acquired microglia characteristics over time, including ramified morphology, longevity, radio-resistance and clonal expansion, but even after prolonged CNS engraftment yolk sac-derived host microglia and BM-derived macrophage transcriptomes and chromatin accessibility landscapes remained distinct[237]. Furthermore, they reported a strikingly similar gene signature of microglia and BM-derived cells when compared to our own data. As in our study, expression of *Ms4a7* and *Clec12a* were reported as being BM engrafted cell-specific, whereas *P2ry12, Sall1* and *Sall3* are only expressed by host microglia even after prolonged integration of BM cells into the parenchyma. The differences in gene expression signatures were partly explained due to the absence of *Sall1* in donor cells, whose transcriptome overlapped significantly with that of *Sall1*-deficient microglia.

Similarly, transplantation studies were used to elucidate similarities and differences between host microglia and BM-derived cells[238]. Even though proper engraftment of donor cells from yolk sac, fetal liver and bone marrow origin were all observed, only yolk sac microglia-like cells expressed a microglia-specific gene signature whereas other cells (fetal liver derived, BM derived) showed similar gene expression to microglia but failed to become 'true' microglia. *P2ry12*[237] and *Ms4a7*[238] were also identified in human tissue as being microglia- and donor cell-specific, respectively, and thus studies of microglia replacement therapies on human tissue will be facilitated by use of these markers.

All-in-all, peripheral infiltration can occur even under homeostatic conditions, but the exact factors need to be further elucidated. A combination of all of the above is likely to occur, depending on the model or disease setting. The brain is a highly dynamic organ and it is likely that there is not only one true explanation of when and how peripheral repopulation occurs or when microglia are able to fill an empty niche through hyper-proliferation.

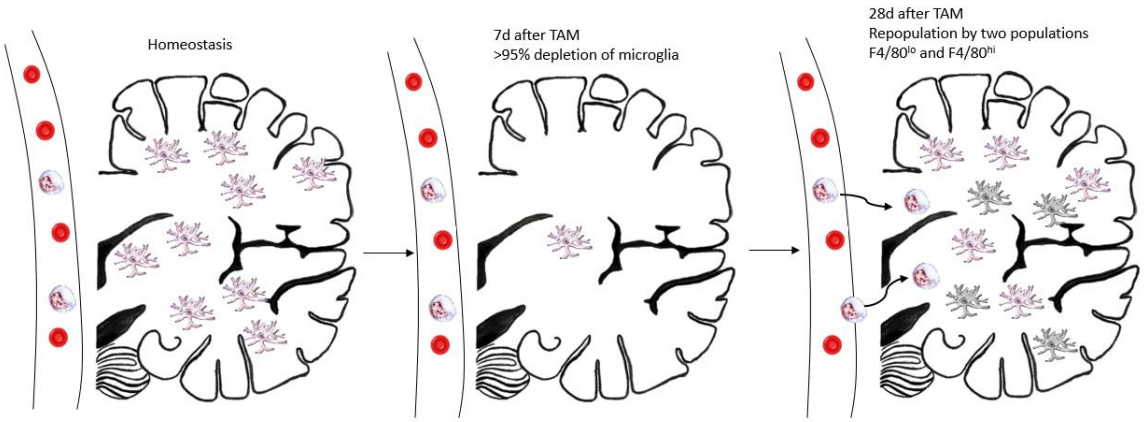

Figure 5: Graphical representation of microglia depletion and repopulation. 7 days after Tamoxifen treatment microglia are depleted >95%. Within 28 days the microglia niche is repopulated by proliferation of resident microglia (F4/80^{lo}, *pink*) and infiltration of Ly6C^{hi} monocytes from the periphery (F4/80^{hi}, *grey*).

3.2 PAPER II: FATAL DEMYELINATING DISEASE IS INDUCED BY MONOCYTE-DERIVED MACROPHAGES IN THE ABSENCE OF TGF-B SIGNALING

Aim:
To study the role of TGF-β in microglia niche repopulation by peripheral cells.

Background:
TGF-β regulates the development and homeostasis of several tissue resident macrophage populations, including microglia. Even though TGF-β is not critical for microglial survival, it is required for the maintenance of the microglia-specific homeostatic gene signature.

Hypothesis:
TGF-β signaling is crucial for niche homeostasis and required for monocyte-derived macrophage integration into the niche.

Results:
Upon deletion of TGF-β signaling in CX3CR1^{+} monocyte-derived macrophages in a setting of CNS repopulation, mice develop a progressive and fatal demyelinating motor disease characterized by myelin-laden giant macrophages throughout the spinal cord and at the end stage in the brain. *Tgfbr2*-deficient macrophages showed high expression of genes encoding proteins for antigen presentation, inflammation and phagocytosis. TGF-β signaling is therefore crucial for the functional integration of monocytes into the CNS microglial niche.

In **paper II** we used findings of paper I to elucidate the role of TGF-β signaling in brain-engrafting macrophages. TGF-β signaling was identified as one of the major regulators of various genes identified in the two different populations of microglia (F4/80^{low}) and brain macrophages (F4/80^{high}). *Tgfβr1* expression in repopulated cell populations was comparable to naïve microglia, whereas it was almost absent in Ly6C^{hi} monocytes. Upregulation of *Tgfβr1* expression upon infiltration into the brain itself hence suggests an important role of TGF-β signaling in brain-engrafting macrophages and microglia. In addition, TGF-β signaling was previously identified to be crucial for the development and maintenance of microglia using RNA-sequencing and functional studies[239].

To address whether TGF-β signaling is crucial for peripheral monocytes to be recruited to the brain, we used bone marrow chimeras in which we reconstituted irradiated *Cx3cr1*^{CreER}-*R26*^{DTA} mice with *LysM*$^{Cre/+}$*Tgfβr2*$^{fl/fl}$ bone marrow. Upon depletion, monocytes lacking TGF-β signaling failed to migrate to the brain or were not able to populate the niche and compete with monocytes with intact TGF-β signaling, hinting towards a crucial role of TGF-β in repopulating-engrafting monocytes. We then investigated what happens when TGF-β signaling is disrupted after monocytes passed the BBB and entered the parenchyma.

We made use of the fact that upon crossing the BBB monocytes start upregulating certain microglia-specific genes, one of them being *Cx3cr1*. We made bone marrow chimeras, transferring *Cx3cr1*^{CreER}*Tgfβr2*$^{fl/fl}$ bone marrow into *Cx3cr1*^{CreER}*-R26*^{DTA} microglia depletion mice. Using this set up we were able to deplete microglia and delete *Tgfβr2* in macrophages that crossed the BBB and upregulated CX3CR1 upon doing so. Surprisingly, mice developed a progressive and fatal demyelinating disease characterized by fore- and hind leg weakness, incontinence and complete paralysis at the end stage. Histological analysis of spinal cord and brain sections revealed that infiltrating macrophages start 'attacking' the spinal cord (and at the end stage of the disease the brain) and ingest myelin. *Tgfβr2* deleted macrophages were transcriptionally characterized by upregulation of genes involved in antigen presentation, inflammation and phagocytosis.

Butovsky *et al.* reported a crucial role of TGF-β signaling in microglia, where mice lacking TGF-β1 in the CNS have defects in extracellular glutamate homeostasis and synaptic plasticity and develop motor symptoms at 4-6 months of age, noticeable by a decreased body weight and reduced rotarod performance[239]. No pathological mechanism was reported, but the authors noted a loss of microglia in these mice in both the brain and the spinal cord. Applying our knowledge from **Paper II**, we can propose a pathological mechanism for Butovskys' findings.

It is not surprising that disruption of TGF-β signaling in microglia and infiltrating macrophages has devastating effects since it was previously reported that mice homozygous for a mutated TGF-β1 allele or TGF-β null develop multifocal inflammatory disease[240] and die soon after birth[241]. Conversely, deleting *Tgfβr2* in myeloid cells in the periphery enhances their anti-tumor abilities by increasing pro-inflammatory genes and antigen presentation genes [242], highlighting the importance and opposing roles of TGF-β signaling in many different diseases. In neurological diseases it was recently shown that TGF-β signaling is often reduced, including Alzheimer's disease [243].

Interestingly, our chimeric mice developed lesions always in the same anatomical regions, starting in the dorsal column of the spinal cord and in the end stage lesions in thalamus, whereas the ventral column of the spinal cord and the cerebrum appeared normal. Regional specificity has been reported in neurological diseases but the origins are still unknown. It would therefore be important to elucidate what determines this very specific demyelination pattern. One step towards answering this question was provided by Grabert *et al.* and Stevens *et al.* who reported that microglia express region-specific gene signatures[244,245].

In conclusion, TGF-β signaling is a central player for the recruitment of macrophages to the brain and their functional integration into the empty microglia niche. The failure to respond to TGF-β may explain the onset or progression of neurodegenerative and neurological diseases.

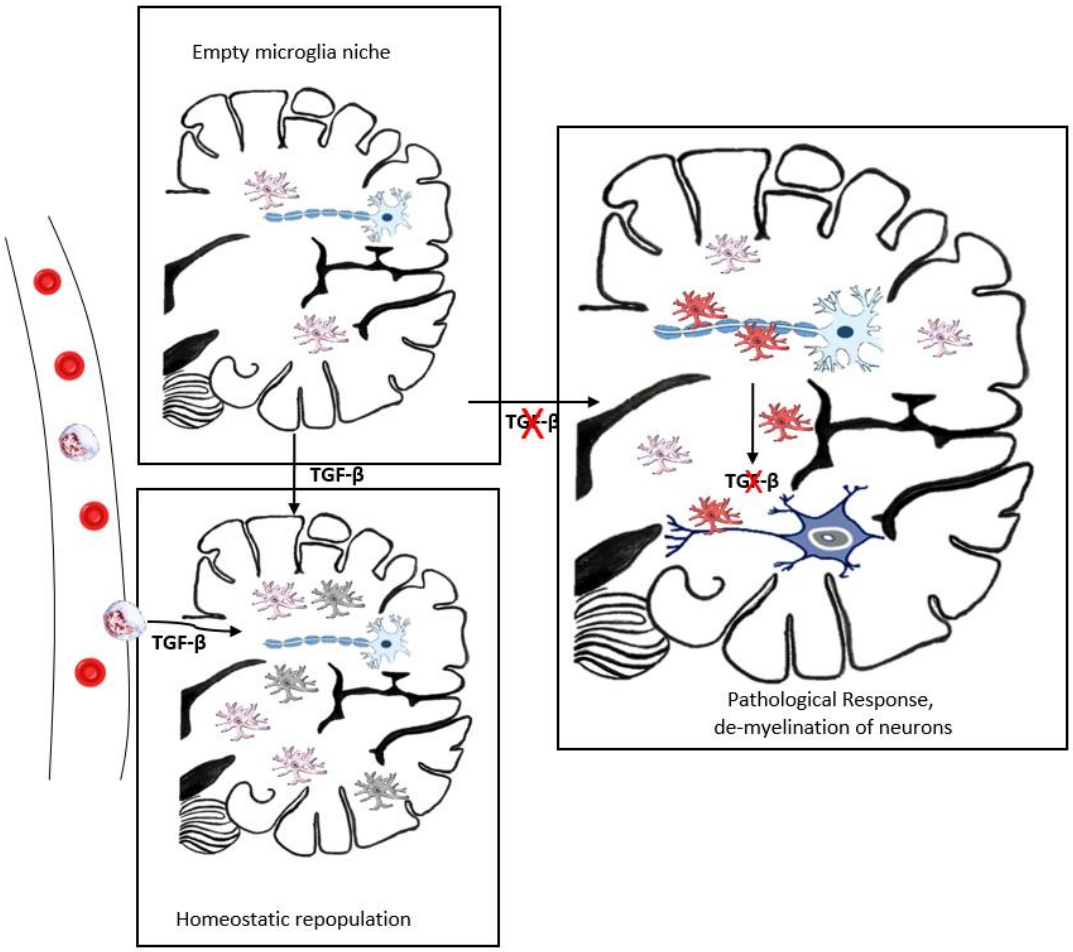

Figure 6: TGF-β regulates integration of monocytes into the empty microglia niche. TGF-β is needed to maintain homeostasis as well as repopulation by monocytes after depletion *(grey)*. In the absence of TGF-β signaling, monocyte-derived macrophages *(red)* attack the tissue by ingesting myelin from axons of neurons *(blue)*.

3.3 PAPER III: EOSINOPHIL DEPLETION AS A NOVEL TREATMENT IN A MOUSE MODEL OF GLIOBLASTOMA MULTIFORME

Aim:
To characterize immune cell infiltration in experimental GBM and identify novel targets for immunotherapy.

Background:
Glioblastoma multiforme (GBM) is a devastating brain tumor with a very short life expectancy after diagnosis. Treatment options are limited to chemotherapy, radiotherapy and surgery, all of which do not significantly prolong survival. Immune cells infiltrate the tumor, with macrophages and microglia reportedly being the major cells contributing to the tumor. Targeting macrophages has been proposed as a treatment but to date no therapy has yielded significant effects on survival.

Hypothesis:
Eosinophils contribute to tumor growth and depleting them will prolong survival.

Results:
Infiltrating monocytes and microglia were both significantly enriched in the tumor, as previously reported. Despite employing radical depletion models for monocyte and microglia depletion we could not significantly prolong survival in the GL261 model. We identified eosinophils as the major cell infiltrate in experimental GBM. Depleting eosinophils using an anti-IL-5 antibody significantly prolonged survival. Eosinophil infiltration was also observed in human GBM tissues, supporting our notion that targeting this usually neglected cell type might be of advantage in the treatment of GBM.

In **Paper III** we studied immune cell infiltration in a mouse model of GBM. In support of the current literature[246,247] we recorded a vast increase in macrophage/microglia number over the time of tumor growth, which led to our first hypothesis of using pro-inflammatory macrophages to counteract the immunosuppressive microenvironment of the tumor, leading to an immune reaction that limits tumor growth. Different targeting strategies to counteract TAMs have been proposed, from inhibition of macrophage recruitment to the tumor[248,249,250,251], suppression of TAM survival[252], enhancing tumoricidal activity of proinflammatory TAMs[253] or blockage of tumor growth promoting activity of immunosuppressive TAMs[254,255].

In a previous study, the Harris lab reported that adoptive transfer of *ex vivo* stimulated M2 macrophages prevents Type 1 diabetes in mice[256]. We decided to use a similar approach to test our hypothesis, by stimulating macrophages *ex vivo* to a pro-inflammatory M1 phenotype and injecting them into the tumor vicinity. Injected macrophages were not able to survive and hence no anti-tumor effect was achieved using this approach.

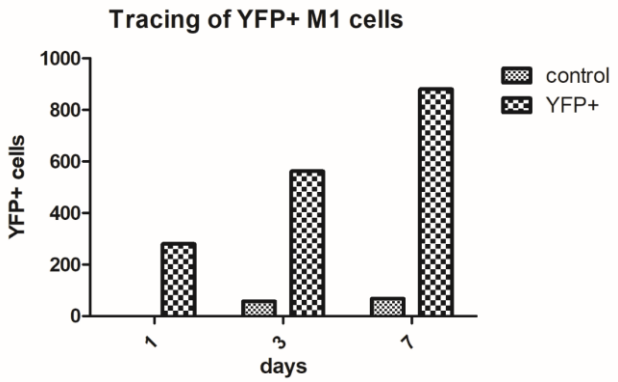

Figure 7: Injection of 150,000 YFP^{+} macrophages into the brain parenchyma. Only a minimal number of M1 macrophages survive upon injection into the tumor vicinity in the brain parenchyma, and no tumoricidal effect was noted.

We therefore revised our approach and targeted monocytes and microglia by using genetic depletion models (*CCR2*$^{-/-}$ mice and *Cx3cr1*^{CreER}-*R26*^{DTA} mice established in **Paper I**). The targeting of single immune cell subsets (monocytes or microglia) only revealed a minimal effect on survival of tumor bearing mice, suggesting that a combination therapy might be a more effective approach for the treatment of GBM. Indeed, targeting CCR2 using antibody treatment by itself was reported to only modestly prolong survival, although significantly in mice. Combination treatment with TMZ increased the effect of CCR2 treatment[249].

Even though we observed significant monocyte/macrophage and microglia numbers in our GBM model, the most abundant cell type was eosinophils. Since targeting monocytes and microglia did not yield significant effects on survival we decided to revise our hypothesis and target eosinophils instead of focusing on macrophages. We identified stable eosinophil accumulation in mouse GBM as evidenced using both flow cytometry and immunohistochemistry. The signals attracting eosinophils to the brain tumor remain to be elucidated. It has been suggested that eosinophil migration is increased by immunosuppressive macrophages due to increased levels of IL-10 and hence increased production of CCL24 by these cells[257]. TAMs usually display an immunosuppressive phenotype and produce IL-10, supporting the idea that TAMs are responsible for eosinophil attraction to the tumor.

Not only immunosuppressive TAMs can attract eosinophils by producing eosinophil chemoattractants, but also signals released from areas of necrosis in the tumor were reported to attract eosinophils[117]. Both mechanisms could provide an explanation for eosinophil recruitment to the tumor in our model. CCR3 staining of tumor brain tissues revealed eosinophilic degranulation in necrotic parts of the tumor.

This could indicate that eosinophils actively try to attack the tumor to a certain extent, but ultimately fail to be effective in this process.

Using an anti-IL-5 antibody (TRFK5)[131] we were able to significantly prolong survival of tumor bearing mice. Treatment with TRFK5 inhibits eosinophil release from the bone marrow into the circulation, but eosinophils already in the circulation are not affected. This could be the explanation why a small number of eosinophils is still present in the tumor, even after depletion. Again, a combination of different antibodies should be considered to achieve a more significant effect on survival.

In human GBM, eosinophil accumulation has so far only been attributed to peri-tumoral edemas, and to our knowledge have not been shown to accumulate in bulk tumor tissue. Dexamethasone, a corticosteroid, is commonly used for the treatment of asthma to prevent eosinophil infiltration into the lungs. It is also implicated in the treatment of peri-tumoral edemas in GBM, which led to reduced tumor size as evidenced by contrast enhanced scans in some cases. This effect is attributed to blockage of GM-CSF release from T-cells by dexamethasone, which decreases survival of GM-CSF-dependent eosinophils[258]. Increase of eosinophil death and consequent release of their cytotoxic granule proteins leads to a short-term reduction in tumor size in GBM patients[259,260,261].

Supportive of our data are several studies of expression of eosinophil chemoattractants and their receptors in human GBM[262], but no studies to date have been performed on eosinophil infiltration into the tumor mass.

In conclusion, we demonstrate a stable eosinophil infiltration in a mouse model of GBM over the time of tumor growth, as well as human GBM tissue, evidenced by immunohistochemistry. The exact mechanism of eosinophil infiltration into the tumor remains to be investigated. We thus suggest that targeting eosinophils directly in GBM patients by blocking eosinophil development or chemotaxis could increase survival and lessen tumor burden.

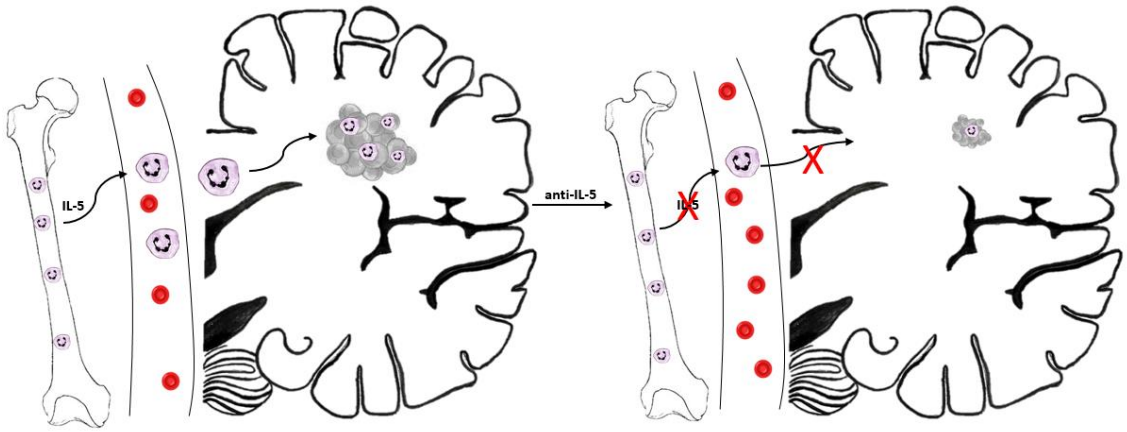

Figure 8: IL-5 is crucial for eosinophil emigration from the bone marrow into the circulation. From the blood, eosinophils travel to the brain and associate with the tumor *(grey)*, due to so far unknown factors. Treatment with anti-IL-5 antibody inhibits eosinophil emigration from the bone marrow, leading to less eosinophil infiltration in the tumor and hence longer survival.

3.4 PAPER IV: ASSESSMENT OF NANOPARTICLE SIRNA DELIVERY *IN VITRO* AND *IN VIVO* FOR THE TREATMENT OF TUMORS IN MOUSE MODELS OF MELANOMA AND GLIOBLASTOMA MULTIFORME

Aim:
To prove the principle of siRNA delivery via nanoparticles for treatment of experimental melanoma and GBM.

Background:
Delivery of siRNA molecules has proven to be difficult due to unspecific release of siRNA and accumulation in tissues.

Hypothesis:
siRNA-loaded nanoparticles can be used to more directly and efficiently target tumor cells and tumor associated immune cells with less adverse effects on healthy tissue.

Results:
We assessed the efficacy of si-TGFβ1, si-IL10 and si-TGFβR2 in either chloroquine-containing nanoparticles or β-glucan encapsulated yeast particles (GeRPs) to modulate macrophage activation *in vitro* as well as the effect on tumor growth *in vivo*. All targets could be significantly down-regulated using siRNA, alone or in combination. GeRPs were readily detectable *in vitro* and *in vivo* using flow cytometry and immunohistochemical approaches. Preliminary *in vivo* data showed promising retardation of tumor growth using chloroquine-siRNA-HA particles in a mouse model of melanoma, making both types of nanoparticles promising new technologies for future cancer treatments.

Since injecting pro-inflammatory M1 macrophages was not successful due to space restrictions and death of the cells, we aimed for a different approach to modulate the tumor microenvironment in **Paper IV**.

Therapies based on RNA interference (RNAi) have been shown to be a powerful method for inhibiting disease targets from any molecular class and numerous proof-of-concept studies have been performed and been successful in various different diseases[263]. siRNAs are macromolecules that need to be delivered to the cellular machinery responsible for mRNA degradation to be effective, but so far there are problems of non-specific accumulation in tissues, poor cellular uptake or inefficient release of siRNA into the cytoplasm[264]. One of the major challenges is therefore successful and targeted drug delivery, to make siRNA drugs more potent and to avoid side-effects. In **Paper IV**, we worked with different nanoparticle (NP) formulations to package siRNA for the treatment of melanoma, and at later stages Glioblastoma multiforme.

TGF-β1 and Tgfβr2 as Targets for siRNA:

TGF-β signaling is involved in many different processes, having crucial roles in development, homeostasis and disease. TGF-β signaling downregulation in myeloid cells has been reported to reduce tumor burden in mice[242,265,266], and hence this signaling pathway is ideal to target for proof-of-concept studies and to determine delivery efficiency of siRNA by nanoparticles. Efficient downregulation of TGF-β1 was achieved *in vitro* in BMDM, B16 melanoma cells and *in vivo* in melanoma tumors, and *in vitro* for *Tgfβr2* in BMDM. Downregulation of TGF-β1 was accompanied with less tumor burden in mice.

GeRPs:

Glucan nanoparticles were specifically developed to target phagocytic cells, mainly macrophages[264]. The β-glucan facilitates specific binding to macrophage-expressed CLEC receptors, thereby ensuring specific delivery to macrophages and not to other immune cells. Following phagocytosis, glucan particles reach acidified endosomes, leading to release of their siRNA into the cytoplasm and allowing specific gene silencing. Previous studies demonstrated that these particles effectively deliver siRNA to various tissue macrophages and that they were effective for the treatment of Diabetes[267], but have so far not been tested in tumor models. Targeting TAMs instead of tumor cells directly was proposed to be a feasible therapeutic approach due to the vast number of TAMs apparent in solid tumors[268].

We confirmed uptake of GeRPs into bone marrow-derived macrophages (BMDM) and efficient downregulation of our target genes. In addition, *in vivo* uptake was confirmed by injection of FITC-labelled GeRPs into the brain parenchyma. FITC signal could be detected for at least 72h after GeRP injection into the brain. Due to its isolated location the brain is ideal for tracing experiments. In addition, trauma caused by the needle leads to macrophage infiltration into the brain. These infiltrating macrophages usually exhibit M1 phenotypes and can be distinguished from microglia by their higher CD45 expression. Infiltrating macrophages phagocytosed significantly more GeRPs than did microglia in the brain at the 24h time point, confirming our *in vitro* phagocytosis assay results in which M1 polarized BMDM were better in phagocytosing GeRP than were M2 cells. This can be explained by the fact that infiltrating macrophages express high levels of *Clec-2*[214], a member of the Dectin-1 family of receptors that is responsible for uptake of β-glucan[269,270]. This is important to consider for further *in vivo* studies in tumor models, since TAMs usually exhibit a M2 phenotype.

CQ-siRNA-HA, Dox-siRNA-HADA:

Activation of the innate and adaptive immune systems by complement activation triggered by opsonin binding, is considered as one of the major obstacles with nanoparticle formulations[271]. As a second class of nanoparticles we used CQ-siRNA-HA and Dox-siRNA-HADA particles. Hyaluronic acid (HA) is a natural polymer derived from the extracellular matrix (ECM) that offers great benefits in engineering NPs owing to their unique biocompatibility, cell surface CD44 receptor targeting ability and non-toxic degradation profiles.

The CD44 targeting ability of HA makes it ideal for developing NPs for anti-cancer therapies, as CD44 receptors are over-expressed in several solid tumors (≈4–5-fold), including glioblastoma multiforme (GBM)[272,273].

In addition to HA, chloroquine in these particles facilitates a modulation of intracellular pH in myeloid cells, which improves the efficiency of tumor immunotherapy[274].

The intrinsic anti-tumor activities of chloroquine by enhancing an M1 phenotype in TAMs, in addition to siRNA delivery to TAMs as well as tumor cells, makes the CQ-siRNA-HA particles a powerful tool for cancer therapy. In a first trial experiment we could significantly reduce tumor burden in B16 melanoma-bearing mice.

In conclusion, **Paper IV** is a proof-of-concept study determining the efficiency of siRNA delivery by nanoparticles of different formulations. CQ-siRNA-HA particles showed promising results in a first *in vivo* trial, and it has to be elucidated whether GeRPs can be as effective considering they only target TAMs in the tumor and not, as CQ-siRNA-HA particles, TAMs and tumor cells at the same time. In addition, even though TGF-β1 was efficiently downregulated in the tumors, treated mice still had quite big tumors, leading to the conclusion that targeting only one gene will not be sufficient to eradicate the tumor. Hence, as already discussed for **Paper III**, a combination of targets will most likely be needed to not only reduce tumor growth but to completely eradicate the tumors.

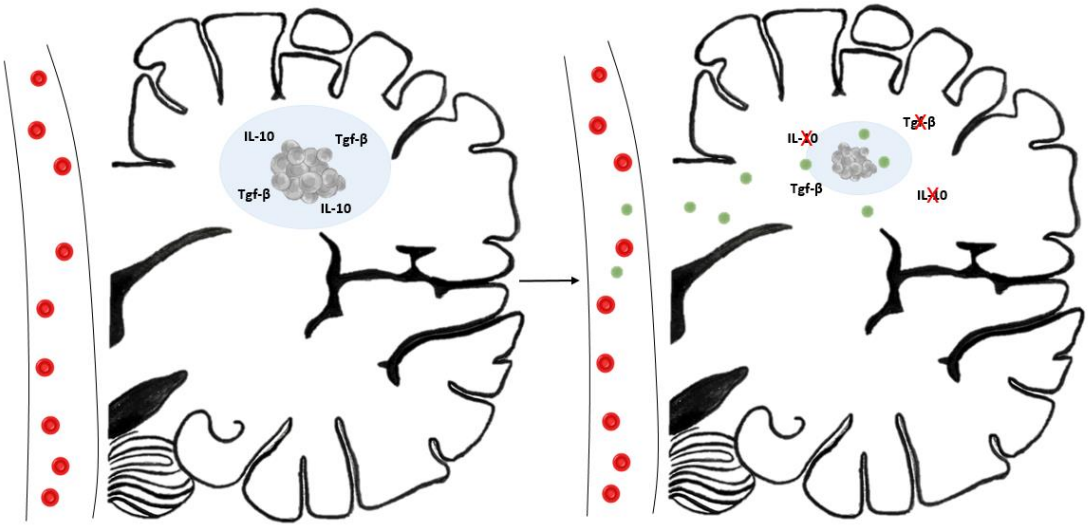

Figure 9: The tumor is surrounded by an immunosuppressive microenvironment *(blue)*, keeping immune cells from targeting the tumor *(grey)*. siRNA-loaded nanoparticles *(green)* against TGF-β or other immunosuppressive cytokines can re-program the tumor microenvironment, leading to an effective immune response and retarded tumor growth.

4 ACKNOWLEDGEMENTS

I want to thank my main supervisor, **Bob** for supporting me in everything I did, be it work related or private. Thank you for the countless reference letters you wrote on my behalf and for supporting my decision to defend early. Your constant motivation, selflessness and ambition made me the scientist I am today and I am grateful having gotten the chance to work in your group for the last 3.5 years.
I want to thank my co-supervisor **Xing-Mei** for sharing her technical know-how and for always having an open ear. Your optimism and great sense of humor made life in the lab so much easier. I also want to thank **Roham** for guiding me not only in my projects but also how to get the most out of my PhD time, even after you left the lab.
A big thanks to **Odd Harald** for including me in your project(s) right from the very beginning of my PhD. Thank you for teaching me basically everything I know about microglia depletion, chimeras, FACS, and so much more. Without you I would not be able to defend early and I am forever grateful for your support.
I am grateful to **Keying** and **Jinming** for discussing experimental procedures and helping me out when I forgot to write stuff down, without you everything would have taken a lot longer to do.

I want to thank the most important person in the lab for me, **Susi**. I knew right from the start choosing you as my mentor would pay off and I want to thank you for your endless advice on absolutely everything, and also painting the cover and figures for my thesis. Thanks for always having an open ear, always being reasonable and guiding me to be a bit of a less stressed out person.

Thanks to **Ewoud** for being an amazing friend and colleague and for correcting various motivation letters over the years. Our lunches will never be forgotten.
I am grateful for all the input on my projects from the people in the PhD room: **Eliane**, **Marie**, **Lara**, **Karl**, **Rasmus** and **Rux**.

A great thank you to **Tomas** and **Mohsen** for running the lab so smoothly and **Gunn** for managing my many, many last minute orders. I am grateful to the animal staff at AKM for taking amazing care of our mice, especially **Elisabeth Qvist, Freja Engström, Sandra Mezei** and **Sandra Olsson**.

I want to thank **Elizabeth Jacobsen** for sharing antibodies, expertise and performing valuable stainings for the tumor projects.

I want to thank the rest of the **Neuroimmunology Unit** for their input and support over the years; I am going to miss working with such great people.

Last but not least I want to thank my **family** for their encouragement and reassurance over the years and I want to specially thank **Alex** for putting up with me during stressful times and when I had to work weekends – this will not change anytime soon and I appreciate your endless patience.

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